

Infiltration of Natural Caries Lesions in Relation to Their Activity Status and Acid Pretreatment in vitro

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Key Words

Enamel caries · Hydrochloric acid · Lesion activity · Nonoperative treatment · Phosphoric acid

Abstract

This study aimed at testing how active and inactive enamel caries lesions differ by their degree of resin infiltration, and whether the choice of acid pretreatment plays a crucial role. Four examiners assessed 104 human molars and premolars with noncavitated enamel lesions and classified them as 'active' or 'inactive' using the Nyvad criteria. Forty-five teeth were included in this study after independent unanimous lesion activity assessment. Lesions were cut perpendicularly into 2 halves. Each half lesion was pretreated with either 15% hydrochloric acid or 35% phosphoric acid. The lesions were infiltrated after staining with rhodamine isothiocyanate. Thin sections of 100 µm were prepared and the specimens were bleached with 30% hydrogen peroxide. The specimens were then counterstained with sodium fluorescein, subjected to confocal laser scanning microscopy and analyzed quantitatively. Outcome parameters were maximum and average infiltration depths as well as relative penetration depths and areas. In active lesions no significant difference of percentage maximum penetration depth and percentage average penetration depth between lesions pretreated with hydrochloric or phosphoric acid could be observed. In inactive lesions, however, phosphoric acid pretreatment resulted

in significantly lower penetration compared to hydrochloric acid pretreatment. Surface conditioning with hydrochloric acid led to similar infiltration results in active and inactive lesions. Moreover, inactive lesions showed greater variability in all assessed infiltration parameters than did active lesions. In conclusion, caries lesion activity and acid pretreatment both influenced the infiltration. The use of phosphoric acid to increase permeability of the surface layer of active lesions should be further explored.

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White spot lesions of the enamel are the first clinical signs of dental caries. Depending on the site-specific state of caries activity, the lesion might either tend to progress ('active lesion') or to stagnate or regress ('inactive lesion') [Manji et al., 1991]. Net progression of a lesion may over time lead to cavitation of the enamel surface, which makes operative intervention necessary if the cavitated area cannot be cleaned by the patient. According to clinical evidence, the risk of cavity formation is substantially higher for active lesions than for inactive lesions [Nyvad et al., 2003], which have been described to remain stable over decades [Backer Dirks, 1966]. Assessment of the activity status of caries lesions is therefore a crucial point for treatment planning [Nyvad et al., 2008].

Traditional preventive measures aim at inactivating active noncavitated enamel lesions by enhancing 'remin-

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eralization'. This might be achieved by better oral hygiene, including the daily use of a toothbrush and fluoride toothpaste. Professional application of high-concentrated fluoride products may boost a positive outcome [Ellwood et al., 2008; Marinho, 2008]. This strategy requires a good compliance by the patient. Patients, however, often show multiple active lesions as a consequence of a lack of compliance and neglect of oral hygiene. Thus, a more invasive chairside method has recently been suggested. This method is the so-called 'infiltration' technique, which aims at instantaneous lesion inactivation of noncavitated enamel lesions by infiltrating the porous carious enamel with a specific resin. The infiltration method was introduced in the last decade and has been tested in vitro on artificial caries of bovine teeth [Paris et al., 2006, 2007a; Meyer-Lueckel and Paris, 2008b] and on natural white spot lesions of human teeth in vitro [Paris et al., 2007b; Meyer-Lueckel and Paris, 2010]) and in vivo in adults [Paris et al., 2010; Martignon et al., 2012; Meyer-Lueckel et al., 2012] and children [Ekstrand et al., 2010].

Lesion inactivation by infiltration is supposed to be achieved by impregnation of the lesion body up to a depth of 620 µm [Paris et al., 2009; Meyer-Lueckel and Paris, 2010] whereby dissolution of enamel by bacterial acids is hampered [Paris et al., 2010]. Importantly, it was demonstrated that in order to obtain successful infiltration of the lesion body the relatively hypermineralized surface layer of the white spot lesions has to be removed by the application of an etching procedure using 15% hydrochloric acid for 90–120 s [Meyer-Lueckel and Paris, 2008b]. Studies reported that only after complete surface layer reduction do capillary forces allow penetration of the infiltrant into deeper parts of the lesion [Meyer-Lueckel and Paris, 2010]. Surface etching with phosphoric acid (20–40%) was tested for resin infiltration in earlier studies using artificial caries models [Gray and Shellis, 2002; Paris et al., 2006] but was found inferior to pretreatment with 15% hydrochloric acid with respect to surface layer reduction [Meyer-Lueckel et al., 2007]. However, little is known about the clinical consequences of complete removal of the mineralized surface layer of incipient enamel lesions. Long-term success of the infiltration technique is likely to depend on the stability of the applied resin over time. In a randomized controlled trial, lesion progression was observed in 4% of the infiltrated proximal initial lesions after 3 years, compared to 42% in the control group [Meyer-Lueckel et al., 2012]. The control group was advised to brush the teeth twice daily with a fluoride toothpaste, to use fluoridated salt and finally to use a fluoridated gel once per week. Similarly, in a

3-year trial it was shown that infiltration or sealing of proximal surfaces had a significantly better therapeutic effect for controlling caries progression than placebo treatment in individuals who received routine instructions on flossing [Martignon et al., 2012]. While these observations suggest that infiltration of approximal caries is a promising method for caries control, there is still some concern that undetected microcavities in the enamel surface [Fejerskov et al., 2008] that cannot effectively be filled by the infiltrant [Paris et al., 2011] might hamper the long-term success of the treatment. It has therefore been proposed that to safeguard the clinical outcome of the infiltration an additional sealant at the surface could be advantageous [Wiegand et al., 2011]. A better acid resistance of infiltrated and additionally sealed artificial lesions was shown compared to infiltrated lesions alone [Schmidlin et al., 2012]. However, although perhaps desirable, it is technically demanding and time consuming to cleanly apply an additional sealant on top of an infiltrated proximal lesion.

Earlier studies of lesion infiltration were performed on lesions confined to the inner half of enamel [Paris et al., 2007b; Meyer-Lueckel and Paris, 2008a], but only recently did the inclusion criteria for white spot lesions in those studies consider their visual appearance ('dull surface, chalky opacity') [Meyer-Lueckel and Paris, 2010; Paris et al., 2011], possibly in an attempt to reflect the status of lesion activity. For purposes of caries control it may only be necessary to infiltrate active lesions, because lesions of this category have a poorer prognosis than inactive lesions [Nyvad et al., 2003]. Moreover, active lesions may be particularly receptive for infiltration because the surface layer of these lesions is more porous than that of inactive lesions [for review, see Fejerskov et al., 2008].

This study had two main goals: to test the method of infiltration in natural white spot lesions that have visually been classified as active or inactive according to the Nyvad criteria [Nyvad et al., 1999] and to test the influence of pretreatment with 35% phosphoric acid or 15% hydrochloric acid on infiltration of active and inactive white spot lesions.

Materials and Methods

Tooth Selection

104 human teeth (premolars, molars) were chosen from a pool of extracted teeth stored in 1% chloramine solution. Informed consent was obtained from the donors to use their teeth for scientific purposes. The chosen teeth showed initial white spot lesions on a proximal surface (ICDAS score 2) [Topping and Pitts, 2009].

Lesion Activity Assessment

The teeth were cleaned and photographed using a digital camera (AxioCam MRC5; Zeiss, Jena, Germany). The printouts of the photographs were used to identify the test site. Four examiners assessed the teeth independently, visually by the naked eye with/without the aid of a stereomicroscope at 6 \times magnification (M5-31660; Wild, Heerbrugg, Switzerland), and scored them as active or inactive according to the visual-tactile criteria of Nyvad [Nyvad et al., 1999]. Lesions with a matt and rough surface were scored active, whereas lesions with a shiny and smooth surface were scored inactive. Only lesions that were unanimously scored as active and inactive by all four examiners were included in this study.

Specimen Preparation

The lesions were sectioned perpendicularly to the surface (Leica SP1600; Leica Microsystems, Wetzlar, Germany), resulting in two lesion halves. The cut surfaces were photographed at 12.5 \times magnification. The cut surfaces and sound enamel were covered by an acid-resistant nail varnish (Maybelline Ultra Lasting; Gemey-Maybelline, Paris, France) leaving a window around the enamel lesions. Paired lesion halves were randomly allocated (www.randomization.com) to one of two etching procedures.

Surface Pretreatment, First Staining and Infiltration

The surface of the carious enamel was pretreated with either 35% phosphoric acid gel (Scotchbond; 3M Espe, Seefeld, Germany) or with 15% hydrochloric acid gel (ICON-Etch; DMG, Hamburg, Germany) for 120 s. The acidic gel was gently moved with a microbrush during application time. Subsequently, the acid gel was washed away during 30 s using a 3-in-1 dental syringe. All teeth were then stored in an ethanolic solution of rhodamine B isothiocyanate (RITC 0.1%; Sigma-Aldrich, Steinheim, Germany) for 12 h as described by Paris et al. [2009]. The lesions were dried by compressed air for 30 s. A commercial infiltrant (ICON-Infiltrant, DMG) was applied on the lesion surface using a microbrush and allowed to penetrate for 3 min. Excess infiltrant material was wiped away with dry microbrushes before light curing for 40 s at 1,200 mW/cm² (Bluephase; Ivoclar Vivadent, Schaan, Liechtenstein). To bleach all red fluorophores from RITC that were not enclosed by infiltrant, specimens were stored in 30% hydrogen peroxide solution for 12 h at 37°C. Subsequently, specimens were washed with tap water for 60 s.

Preparation of Thin Slices and Second Staining

After peeling off the nail varnish, the specimens were remounted in the saw (Leica SP1600; Leica Microsystems) and sections of about 200 μ m were obtained. The sections were polished to a final thickness of 100 μ m using a water-cooled rotating polishing machine (Knuth-Rotor; Struers, Copenhagen, Denmark) with silicon carbide paper up to No. 4,000 grit. To visualize porous structures such as noninfiltrated lesion parts, specimens were immersed in a 50% ethanol solution of 100 μ M sodium fluorescein (NaFl; Sigma-Aldrich, Brøndby, Denmark) for 3 min. Subsequently, specimens were washed in deionized water for 10 s.

Confocal Laser Scanning Microscopy

The thin slices were imaged with an inverted confocal laser scanning microscope (Zeiss LSM 510 META) equipped with a 10 \times objective, 0.3 numerical aperture (EC Plan-NEOFLUAR).

488- and 543-nm laser lines were used for sequential excitation of NaFl and RITC, and fluorescence emission was detected in 501–544 and 597–651 nm intervals (META detector) with the confocal pin holes set to an optical slice thickness of 11.5 μ m. For each lesion, seven equidistant (15 μ m) z-stack images were acquired. Images were 1,024 \times 1,024 pixels (1,273 \times 1,273 μ m²) in size and were acquired with pixel dwell time 1.28 μ s, line average 2, 8-bit intensity resolution. If lesions exceeded the size of one microscopic field of view, multiple images were taken.

Image Analysis

All distance measurements were performed along the axis of the enamel rods (Image J; NIH, Bethesda, Md., USA). Average lesion depth (LD_{av}) was calculated as the mean value of 21 randomly allocated distance measurements in all layers of the z-stack. The maximum LD (LD_{max}) was defined as the greatest distance found between the surface and bottom of the lesion in any of the 7 stack layers. Accordingly, penetration depth (PD) of the infiltrant was measured as maximum (PD_{max}) and average PD (PD_{av}), using the same measurement points as for LD. Furthermore, maximum projections of the acquired z-stacks were produced (Image J), and for every lesion the relative infiltrated area was calculated using the daime software (digital image analysis in microbial ecology) [Daims et al., 2006].

Statistical Analysis

Statistical analysis was performed using SPSS software (PASW for Mac 17.0; SPSS Inc., Chicago, Ill., USA). Differences in LD between active and inactive lesions were calculated using the Wilcoxon signed rank test. To more accurately estimate the infiltration effectiveness, PD_{max} and PD_{av} were calculated relative to LD (%PD_{max}, %PD_{av}). To test the impact of the activity status or acid pretreatment on relative infiltration area, %PD_{max} and %PD_{av}, a nonparametric ANOVA model with repeated measurements, i.e. the F1_LD_F1 model [Brunner et al., 2002], was applied. Post hoc descriptive analyses were applied to compare the acids per activity status and the activity status per acid (Wilcoxon rank sum test). The level of significance was set at 0.05 for all tests.

Results

In the sample of 104 teeth with noncavitated enamel caries, many lesions were 'mixed lesions', containing both active and inactive sites. Twenty-one lesions were unanimously scored as 'typical active' and 24 lesions as 'typical inactive'; thus, the final sample consisted of 45 lesions. No significant differences regarding LD_{max} could be observed for the corresponding lesion halves that were treated with either phosphoric or hydrochloric acid (Wilcoxon signed rank test; $p > 0.05$).

Median values and the corresponding nonparametric 95% CI of LD and PD are given in table 1. Inactive lesions were shallower than active lesions (table 1). However, in inactive lesions the phosphoric acid etching procedure seemed to result in enough open enamel pores to allow

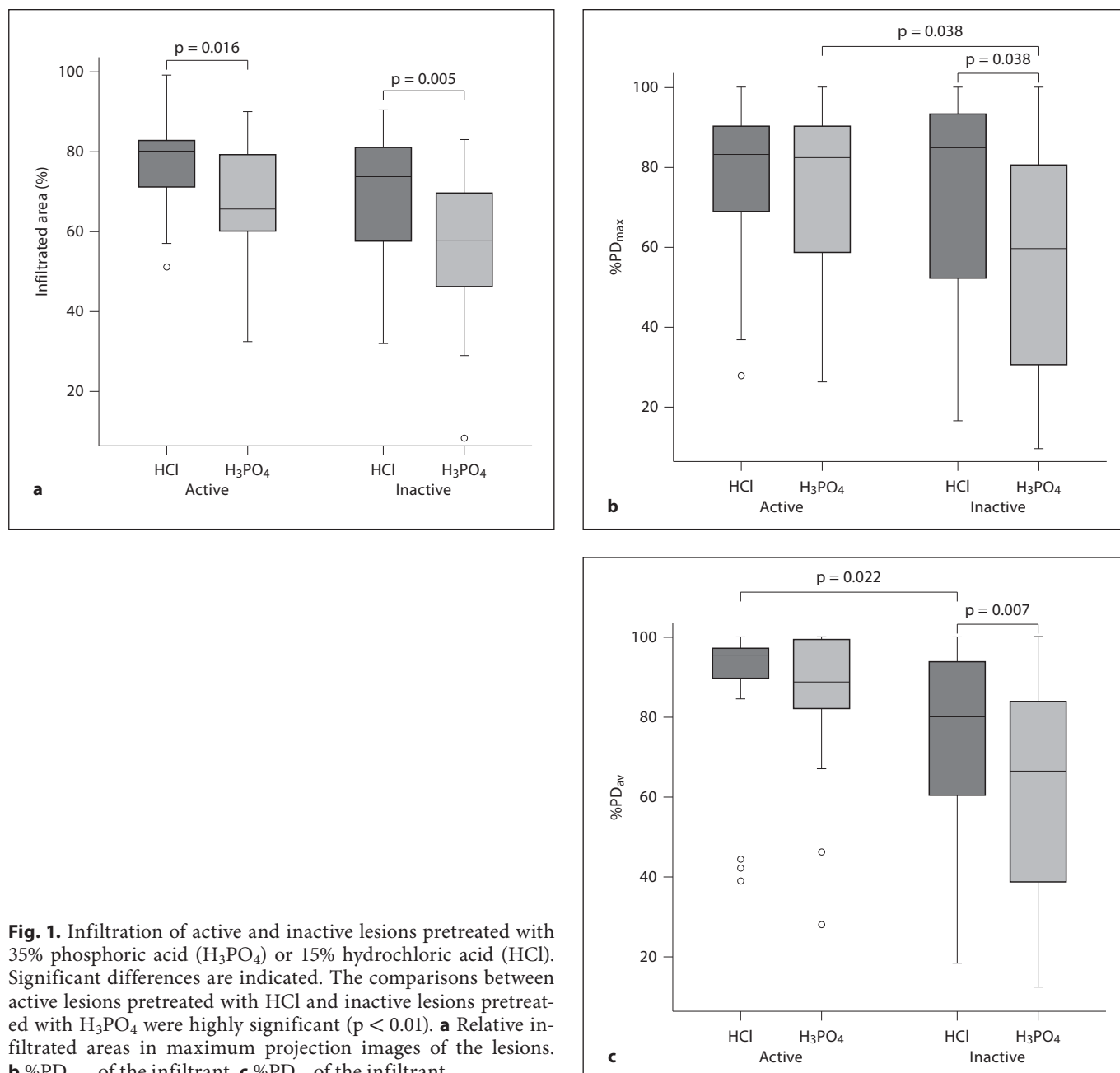


Fig. 1. Infiltration of active and inactive lesions pretreated with 35% phosphoric acid (H₃PO₄) or 15% hydrochloric acid (HCl). Significant differences are indicated. The comparisons between active lesions pretreated with HCl and inactive lesions pretreated with H₃PO₄ were highly significant ($p < 0.01$). **a** Relative infiltrated areas in maximum projection images of the lesions. **b** %PD_{max} of the infiltrant. **c** %PD_{av} of the infiltrant.

infiltration into the lesion body up to about 300 μm or about 60% of the lesion area (table 1; fig. 1a).

Both lesion activity status and the chosen type of acid pretreatment had a significant effect on the relative infiltrated area, while %PD_{max} was significantly influenced by acid pretreatment and %PD_{av} by lesion activity only (table 2). The product factor 'activity:acid' describes the interaction of acid and lesion activity, i.e. whether the ef-

fect of the acid pretreatment is limited to active or inactive lesions. No such effect could be detected (table 2).

For visualization, the quantitative results for %PD_{max}, %PD_{av}, and relative infiltrated areas are presented as box-plots in figure 1. %PD_{max} did not differ between active lesions pretreated with hydrochloric or phosphoric acid and inactive lesions pretreated with hydrochloric acid, but %PD_{max} was significantly lower in inactive lesions

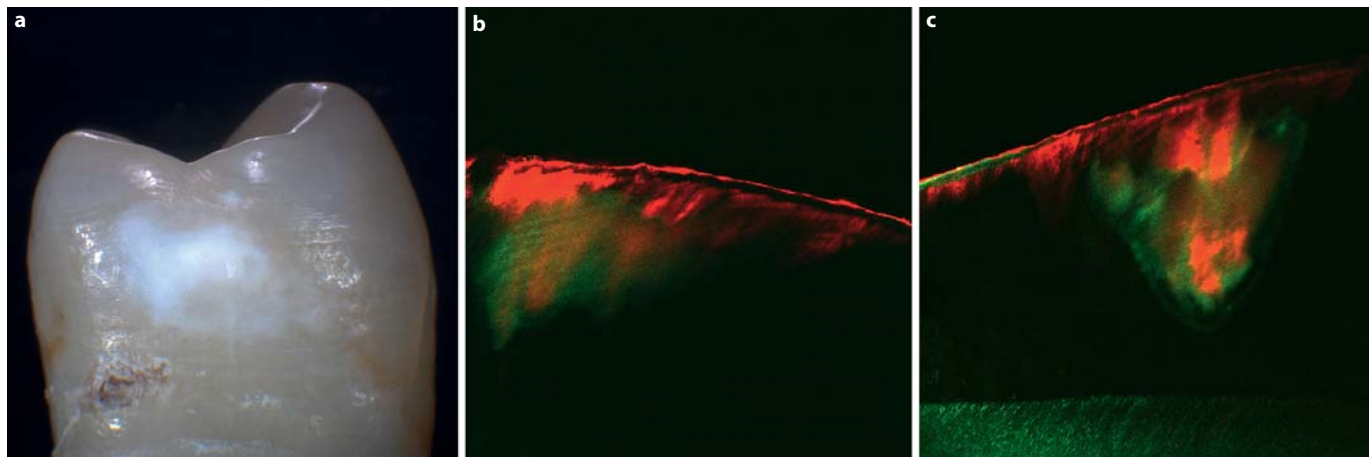


Fig. 2. Lesion unanimously rated as 'active'. **a** Photograph, 12.5× magnification. **b** Lesion half, infiltrated after pretreatment with 15% hydrochloric acid. The infiltrated part of the lesion is stained with RITC (red), while noninfiltrated areas are stained with so-

dium fluorescein (green). **c** Lesion half, infiltrated after pretreatment with 35% phosphoric acid. %PD_{max} and %PD_{av} did not differ between the two acid pretreatments.

Table 1. Median values (and corresponding nonparametric 95% CIs) of LD_{max}, LD_{av}, PD_{max} and PD_{av} of active and inactive white spot lesions

	Active lesions		Inactive lesions	
	HCl	H ₃ PO ₄	HCl	H ₃ PO ₄
LD _{max} , μm	756 (630; 970)	752 (528; 918)	593 (398; 879)	687 (469; 874)
LD _{av} , μm	579 (464; 742)	534 (402; 731)	479 (383; 735)	493 (414; 626)
PD _{max} , μm	549 (376; 815)	475 (303; 763)	340 (290; 539)	296 (251; 342)
PD _{av} , μm	460 (288; 742)	462 (261; 731)	288 (256; 416)	267 (217; 340)

HCl = Hydrochloric acid; H₃PO₄ = phosphoric acid.

pretreated with phosphoric acid. Generally, inactive lesions showed a higher variability than active lesions with respect to all lesion parameters evaluated (fig. 1).

Figures 2 and 3 show representative examples of an active and inactive lesion that were cut in half and pretreated with different acids. Examination revealed that non-infiltrated parts of the lesions were regularly found in the innermost regions of the lesions, adjacent to sound enamel. Furthermore, many lesions showed double stained areas in single planes of a z-stack (fig. 4).

Discussion

This study shows that both lesion activity status and acid pretreatment influence the outcome of infiltration studies of noncavitated caries lesions.

Table 2. Influence of activity status, acid pretreatment and the interaction between acid and activity on infiltration.

Effect	Outcome parameter	p value
Activity	relative infiltrated area	0.0109
	%PD _{max}	n.s.
	%PD _{av}	0.0016
Acid	relative infiltrated area	<0.0001
	%PD _{max}	0.0120
	%PD _{av}	n.s.
Activity:acid	relative infiltrated area	n.s.
	%PD _{max}	n.s.
	%PD _{av}	n.s.

ANOVA-type statistic [Brunner et al., 2002]; degree of freedom = 1.

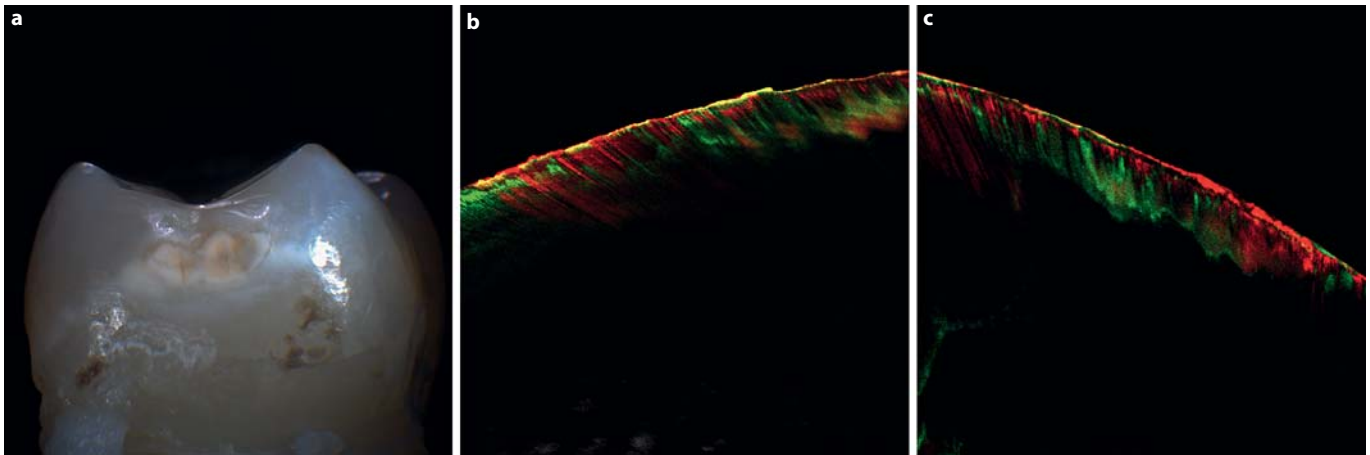


Fig. 3. Lesion unanimously rated as ‘inactive’. **a** Photograph, 12.5× magnification. **b** Lesion half, infiltrated after pretreatment with 15% hydrochloric acid. The infiltrated part of the lesion is stained with RITC (red), while noninfiltrated areas are stained

with sodium fluorescein (green). **c** Lesion half, infiltrated after pretreatment with 35% phosphoric acid. Relative infiltration is smaller than after pretreatment with hydrochloric acid.

The observation that active lesions were better infiltrated than inactive lesions does not come as a surprise. Active enamel lesions have bigger pores and less mineralized surface layers than inactive enamel lesions [Thylstrup et al., 1994; Cochrane et al., 2012]. This allows the infiltrant to penetrate deeper and fill out more of the body of the lesion. An increased area of infiltration and PD_{av} after pretreatment with hydrochloric acid might also be expected since hydrochloric acid removes more of the surface layer than phosphoric acid [Meyer-Lueckel et al., 2007], thereby facilitating access of the infiltrant to the interior of the lesion. In the latter study a systematic differentiation between active and inactive lesions was not carried out. Interestingly, in our sample, it did matter whether lesions were classified as active or inactive. In active lesions there was no significant difference in $\%PD_{max}$ between pretreatment with phosphoric acid and hydrochloric acid, suggesting that surface conditioning with phosphoric acid is sufficient to increase permeability of the surface layer and allow infiltration of active noncavitated lesions. By contrast, surface conditioning with phosphoric acid was less effective than hydrochloric acid in infiltration of inactive lesions. This might probably be explained by the variable and often thick surface layers of inactive lesions inhibiting penetration of acids and acrylate molecules into the lesion [Iijima and Tagaki, 2000; Cochrane et al., 2012], a phenomenon that was also reflected by a high variation in infiltration depths of the inactive lesions in our study. In a clinical perspective, our results may explain why it is more difficult to predict the outcome of postorthodontic mask-

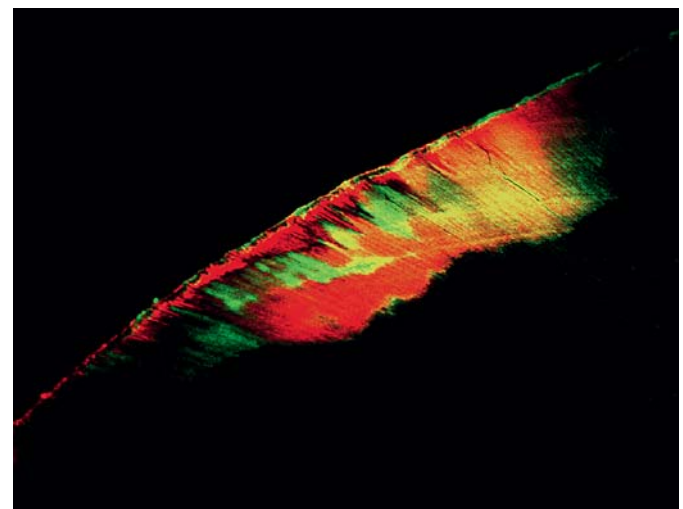


Fig. 4. Single plane of a z-stack (confocal laser scanning microscopy) of an infiltrated lesion. We often noticed yellow areas which indicate double staining with both dyes.

ing of inactive enamel lesions [Neuhaus et al., 2010] compared to active lesions [Paris and Meyer-Lueckel, 2009; Rocha Gomes Torres et al., 2011].

Our study applied area estimates as a response variable. These area estimates are based on maximum projections of z-stacks of about 100- μm thickness of the lesion and differ from those used in an earlier publication [Paris et al., 2011]. Previous studies of lesion infiltration have mostly been evaluated by measuring PD at the deepest site, or

at 'characteristic' landmarks of the lesion in a single reference plane [Paris et al., 2009, 2011]. However, it has not been reported so far whether the depth measurements were taken along the enamel rods or perpendicular to the surface. The effect of acid pretreatment was shown to have a significant influence on %PD_{max} but not %PD_{av}, while lesion activity significantly influenced %PD_{av} but not %PD_{max} (table 2). Area measurements could be more robust than depth measurements because a higher amount of data is included in the estimates. However, because shallow lesions are better infiltrated than deeper ones, area estimates may suggest almost complete penetration while deep lesion parts are only partially infiltrated. We found that area estimates seemed to be less dependent on penetration peaks. Therefore, giving results for both %PD_{max} and relative infiltrated areas is probably the most accurate way to report findings of in vitro infiltration studies.

PDs of the infiltrant of about 500 µm in active lesions are in the range of data reported by other groups. Both lower (264 µm) [Meyer-Lueckel and Paris, 2008a] and higher PDs (626 µm for the experimental resin PC204) [Meyer-Lueckel and Paris, 2010] were reported previously. A lower infiltration depth (<50 µm) was found after 120 s surface pretreatment with 37% phosphoric acid [Paris et al., 2007b]. We found consistently higher infiltration depths also in inactive lesions. This might be attributable to the fact that in the latter study a more viscous resin was used which was labeled with 0.1% tetramethylrhodamine isothiocyanate. Both the resin with the lower penetration coefficient and the direct staining method might contribute to lower PD measurements. The indirect staining method that was used in the current study setup (1st stain, infiltration, bleaching, 2nd stain) showed a generally better reliability [Paris et al., 2009] and allowed more accurate resin penetration measurements that led to higher values [Paris et al., 2011].

Contrary to other studies that reported almost complete LD infiltration [Meyer-Lueckel and Paris, 2010], we only observed incomplete infiltration of about 80% of the area in active lesions and 75% in inactive lesions (fig. 1a). The deepest parts of our noncavitated lesions were typically not infiltrated (fig. 2b, c). Based on comparisons with polarized light microscopic images of incipient enamel lesions these inner parts of the lesions may correspond to the dark zone [Teranaka et al., 1986]. The dark zone has a higher content of organic material (protein and lipids) that may occlude submicroscopic pores [Shellis et al., 2002] and hamper infiltration. A recent study combined polarized light microscopy with fluorescence microscopy, and although assessment of the dark zone was not the primary

aim, one superimposed figure in that publication supports our hypothesis [Arnold and Gaengler, 2012]. However, considering that in the latter study only 5 teeth were included, a firm association between the noninfiltrated area and the presence of a dark zone can only be verified in a combined experimental setup using polarized light microscopy and confocal laser scanning microscopy.

In our study, double stained areas (red and green) could be observed in both active and inactive lesions (fig. 4). One possible explanation for this phenomenon could be prior demineralization/remineralization cycles that led to irregular zones of mineral reprecipitation [ten Cate et al., 2008], hindering complete penetration of the infiltrant and leaving binding sites for NaFl in close proximity to red fluorophores. However, red and green fluorophores might also bind in identical xy positions, but different z positions of an image stack.

In conclusion, within the limitations of this laboratory study, we showed that both lesion activity status and acid pretreatment influence the depth and area of infiltration of natural white spot lesions. Consequently, lesion activity should be considered in future infiltration studies. Acid pretreatment with hydrochloric acid improved the overall lesion infiltration compared to pretreatment with phosphoric acid, especially in inactive lesions. The clinical implications of the latter finding remain speculative and leave room for discussion. From a biological point of view, it may be risky to lose the outer protective layer of an incipient lesion due to acid conditioning because if the infiltrated resin is degraded over time (Van Landuyt et al., 2011) the lesion might be prone to progression. On the other hand, we do not know if full penetration of the infiltrant into a caries lesion is of vital importance for long-lasting clinical results. It could well be (as with occlusal sealing) that a firm surface seal, preventing bacterial acids from reaching the enamel, is sufficient to control lesion progression [Martignon et al., 2012]. Therefore, to protect the natural surface layer of lesions, we recommend that the use of phosphoric acid to increase permeability of the surface layer of active lesions should be further explored.

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K.W.N., A.L. and B.N. conceived and designed the experiments; K.W.N. and S.S. performed the experiments; K.W.N., S.S., A.L. and B.N. analyzed the data; K.W.N., S.S., A.L. and B.N. wrote the manuscript.

Disclosure Statement

None of the authors of the present paper has a conflict of interest.

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